

**THE ROLE OF GUT PERMEABILITY AND SERINE-PROTEASES
IN THE PATHOMECHANISM OF
IRRITABLE BOWEL SYNDROME AND INFLAMMATORY BOWEL DISEASES**

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I. Gecse K, Roka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztoczy A, Izbeki F, Fioramonti J, Wittmann T, Bueno L. Increased fecal serine-protease activity in diarrheic IBS patients: a colonic luminal factor impairing colonic permeability and sensitivity.

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II. M. Dabek, L. Ferrier, R. Róka, K. Gecse, A. Annaházi, J. Moreau, J. Escourrou, C. Cartier, G. Chaumaz, M. Leveque, A. Ait-Belgnaoui, T. Wittmann, V. Theodoru, L. Bueno. Luminal cathepsin G and protease-activated receptor 4: a duet involved in alterations of colonic epithelial barrier in ulcerative colitis.

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LIST OF ABBREVIATIONS

AEBSF: 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride

Cat-G: cathepsin G

CD: Crohn's disease

CPP: colonic paracellular permeability

EMG: electromyography

FITC-dextrane: fluorescein isothiocyanate dextrane

IBD: inflammatory bowel diseases

IBS: irritable bowel syndrome

IBS-D: diarrhea-predominant irritable bowel syndrome

IBS-C: constipation-predominant irritable bowel syndrome

IBS-M: IBS patients with mixed pattern

IBS-U: unsubtyped IBS

INF: acute infectious diarrhea

MLC: myosin light chain

MLCK: myosin light chain kinase

MPO: myeloperoxidase

PARs: protease-activated receptors

pMLC: phosphorylated myosin light chain

PMN: polymorphonuclear neutrophil

SBTI: soybean trypsin inhibitor

SCGI: specific cathepsin-G inhibitor

SLIGRL: (H-serine-leucine-isoleucine-glycine-arginine-leucine-OH)

SLPI: human secretory leucocyte protease inhibitor

SPA: serine-protease activity

UC: ulcerative colitis

ZO-1: zonula occludens-1

SUMMARY

Introduction Inflammatory bowel diseases (IBD) and diarrhea-predominant irritable bowel syndrome (IBS-D) are characterized by elevated colonic luminal serine protease activity. Luminal serine proteases act on protease-activated receptors (PARs), whose activation has been associated with increased paracellular permeability. Defective epithelial barrier has been implicated in the pathogenesis of both in irritable bowel syndrome (IBS) and in IBD.

Aims The studies were conducted (i) to investigate the origin of elevated serine protease activity in IBS-D patients, (ii) to evaluate if it may be sufficient to trigger alterations in colonic permeability and sensitivity in IBS-D, (iii) to examine the possible involvement of PAR-2 activation in this process and (iv) to investigate the underlying molecular mechanisms, (v) to evaluate whether high colonic luminal serine-protease activity in UC may contribute to increased gut permeability and (vi) to examine the possible involvement of cathepsin-G, a neutrophil-derived serine-protease, and PAR-4 in this process. The studies also aimed (vii) to measure intestinal and colonic permeability in vivo in patients with IBS and (viii) with UC in remission, and (ix) to investigate possible correlation between increased gut permeability and clinical symptoms in IBS-D patients.

Patients and Methods Fecal enzymatic activities were assayed in healthy subjects and in patients with IBS, ulcerative colitis and acute infectious diarrhea. Visceral sensitivity was evaluated following mucosal exposure to supernatants from control subjects and IBS patients by recording electromyographic response to colorectal balloon distension in wild-type and PAR-2^{-/-} mice. Colonic paracellular permeability (CPP) was evaluated on murine colonic strips in Ussing chambers. Tight junction protein zonula occludens-1 (ZO-1) and phosphorylated myosin light chain (pMLC) were detected by immunohistochemistry. In vivo gut permeability was evaluated by measuring 24-hour urine excretion of orally administered ⁵¹Cr-EDTA in IBS and UC patients and in control subjects. Clinical symptoms were evaluated in IBS-D patients and correlated to colonic permeability.

Results The threefold increase in fecal serine protease activity seen in IBS-D patients is of neither epithelial nor inflammatory cell origin, nor is it coupled with decreased antiprotease activity of endogenous origin. Mucosal application of fecal supernatants from IBS-D patients

evoked allodynia and increased CPP in mice, both of which effects were prevented by serine protease inhibitors and dependent on PAR-2 expression. Colonic exposure to supernatants from IBS-D patients resulted in rapid phosphorylation of myosin light chain and a delayed redistribution of ZO-1 in murine colonocytes. Mucosal application of UC fecal supernatants increased CPP in mice, an effect that was prevented by a cathepsin-G inhibitor and PAR-4 antagonist. In vivo gut permeability is significantly decreased in the proximal small intestine in IBS-C patients, however distal small intestinal permeability showed no significant difference in the studied group of patients compared to controls. Colonic permeability of IBS-D and inactive UC patients was significantly increased compared to controls. Colonic permeability of IBS-D patients showed correlation with stool frequency.

Conclusions Elevated colonic luminal serine protease activity in IBS-D patients evokes a PAR-2-mediated colonic epithelial barrier dysfunction and subsequent allodynia in mice, suggesting a novel organic background in the pathogenesis of IBS. Increased luminal cathepsin-G may in fact contribute to the development or aggravation of defective epithelial barrier in a PAR-4 dependent pathway in active UC. Elevated gut permeability is restricted to the IBS-D subgroup of IBS patients and is localized to the colon both in IBS-D and in inactive UC patients. Even though the question still persists whether altered barrier function makes a primary or secondary contribution to IBS and IBD pathogenesis, restoring barrier function remains a future therapeutic objective.

INTRODUCTION

The intestinal epithelium is faced with the dual task of providing a barrier while also allowing nutrient and water absorption, therefore its integrity is crucial to maintain physiological function and prevent diseases. Defective epithelial barrier function, which can be measured as increased gut permeability, has been implicated in the pathogenesis of both irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD).

Irritable bowel syndrome is a gastrointestinal disorder characterized by abdominal pain and altered bowel habit, for which there is no apparent structural basis. The diagnosis of irritable bowel syndrome is symptom-based according to the Rome criteria, since 2006 Rome III being the most recent¹. “Red flag” symptoms for organic disorders are ruled out by careful history taking and thorough physical examination. Rome III defines IBS as recurrent abdominal pain or discomfort lasting for at least 3 days per month in the last 3 months, which is associated with 2 or more of the following characteristics: improvement with defecation, onset associated with change in stool frequency or onset associated with change in stool form. Irritable bowel syndrome affects 5–20% of the population worldwide², thus increasing interest has recently been shown towards its poorly understood pathophysiology and to possible therapeutic approaches of the disease. Due to intensive research of recent decades, there has been a paradigm shift in IBS pathophysiology from considering it a purely psychosocial disturbance to finding organic backgrounds of the disease. By now, genetic factors, altered “brain-gut axis” - both in terms of altered sensory afferent function and central processing resulting in visceral hypersensitivity - abnormal serotonergic neurotransmission, altered gut motility, changes in the gut microflora, stress, intestinal hyperpermeability and mucosal immune activation have all been implicated in the pathogenesis of IBS. In a preliminary pilot study, it has been shown that fecal supernatants of diarrhea-predominant IBS (IBS-D) patients have a substantially higher serine protease activity, similar to that of patients with ulcerative colitis (UC), when compared with healthy subjects or other subgroups of IBS patients³. Certain serine proteases are signaling molecules that cleave protease-activated receptors (PARs), a family of G-protein coupled receptors with a widespread distribution (Figure 1).

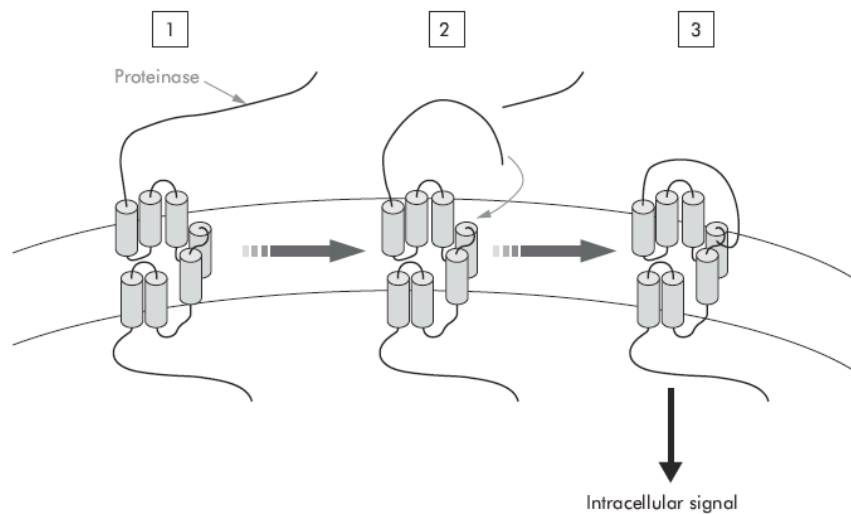


Figure 1. Mechanism of PAR activation. Proteinases cleave the extracellular N terminal domain (1) releasing a new N terminal tail which acts as a “tethered ligand”, that binds the receptor itself (2) to induce an intracellular signal (3)⁴.

Four members of the PAR family have been identified so far in human tissues, of which PAR-2 and 4 are highly expressed on intestinal epithelial cells⁵⁻⁷. The primary activating protease of PAR-2 is trypsin, however, serine proteases of other endogenous and/or bacterial origin are also able to cleave the receptor⁸. Activation of PAR-2 modulates several gastrointestinal functions, such as motility, ionic exchange, paracellular permeability, sensory functions and inflammation⁴. Intracolonic but not intraperitoneal administration of the synthetic selective PAR-2 agonist, SLIGRL (H-serine-leucine-isoleucine-glycine-arginine-leucine-OH), produces delayed visceral hyperalgesia in rats associated with increased paracellular permeability⁹⁻¹¹. In the intact epithelium the paracellular space between adjacent cells is sealed by dynamically changing tight junctions at the luminal aspect of the apical junction complex (Figure 2), which forms a selectively permeable barrier and is structurally related to the perijunctional actomyosin ring. The integrity of the epithelial barrier is dependent upon the contraction of this perijunctional actomyosin ring and subsequent physical tension on the tight junction, an event that involves the phosphorylation of myosin light chain (MLC), initiated by MLC kinase (MLCK)^{12,13}.

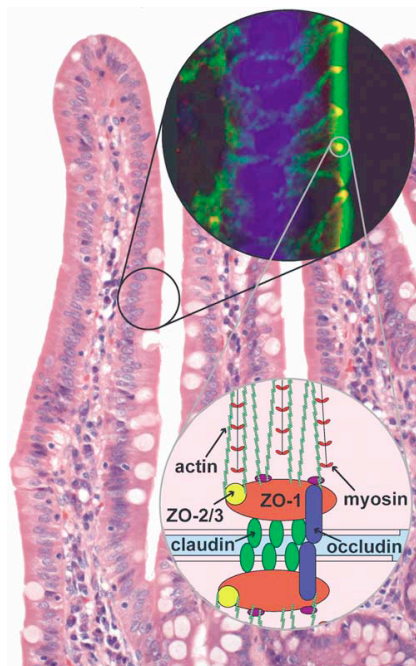


Figure 2. The tight junction. Immunofluorescence demonstrates the restricted location of the tight junction. Nuclei are stained blue, actin is green and zonula occludens-1 (ZO-1) tight junction protein is red; all of the ZO-1 co-localizes with perijunctional actin, producing a yellow color at the tight junction. The claudin family of proteins form the actual paracellular pore within the tight junction, and is associated with another transmembrane protein, occludin. ZO-1, 2 and 3 are attached to this complex¹⁴.

Interestingly, both visceral hypersensitivity¹⁵⁻¹⁷ and impaired intestinal permeability are commonly observed features of IBS. Gut permeability has been reported to be enhanced in 50% of post-infectious IBS patients, in agreement with the study showing increased small intestinal permeability in both the post-infectious and sporadic forms of IBS, characteristically in the diarrhea-predominant subtype¹⁸⁻²⁰, the same subpopulation where increased serine protease activity has been detected³. In accordance, the report on the “Walkerton epidemic” - a waterborne outbreak of acute gastroenteritis in Walkerton, Ontario - proved subtle increase in small intestinal permeability in a large number of patients with IBS, however in vitro studies suggest enhanced permeability in colonic biopsies of IBS patients compared to healthy subjects²¹⁻²². Therefore it seems that gut permeability in IBS is altered, though the data on the subgroup of IBS patients affected and the exact localization of the defective barrier are still contradictory. It is also well established that impaired intestinal barrier function could facilitate the passage of luminal antigens and lead to mucosal immune response²³. Furthermore, there is growing evidence for microinflammation of the intestinal and colonic mucosa to play a role in IBS pathogenesis²⁴⁻²⁷. Thus identifying the role of defective mucosal barrier in IBS pathomechanism and symptom generation may be an important landmark in better understanding of the disease.

Inflammatory bowel disease is a chronic inflammatory condition of the intestines that is characterized by remission and relapses and distills clinically into one of the two major subtypes of disease: ulcerative colitis and Crohn's disease (CD). Current understanding regarding the underlying pathophysiological mechanisms in IBD is that dysregulated mucosal immune response leads to barrier defect triggered by antigenic components of the normal commensal microbiota that reside within the intestine in a genetically susceptible host. Epithelial barrier impairment is considered important in IBD as it leads to increased luminal antigen exposition of the lamina propria, i.e. immune cells, including large number of neutrophils²⁸, which further aggravate both the inflammatory process and the hyperpermeability (Figure 3).

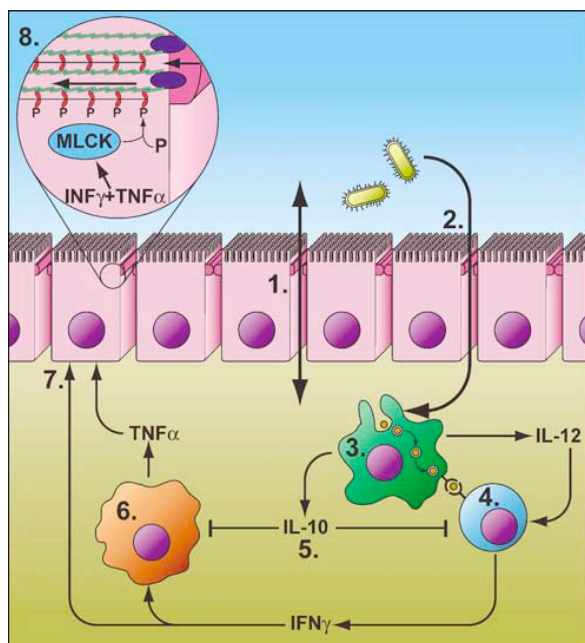


Figure 3. A model for the pathogenesis of inflammatory bowel disease. (1) Initial barrier disruption, which leads to a (2) mixing of luminal content (ie. bacteria) with lamina propria content, ie. antigen presenting cells (3) that promote (4) T-cell response and secretion of the pro-inflammatory cytokine IL-10 (5). T cells respond by secreting IFN γ that induce macrophage activation (6), which in turn secrete TNF α . Both IFN γ and TNF α are known to affect epithelial barrier function (7). Through unknown mechanism, these cytokines activate MLCK (8) leading to MLC phosphorylation, actomyosin contraction and opening of the tight junction, which leads to a vicious cycle in disease progression¹⁴.

Neutrophils are characterized by two major granule populations, primary (azurophil) and secondary (specific) granules, formed at different stages of neutrophil maturation. Cathepsin-G (Cat-G), a serine protease, makes up approximately 20% of the neutrophil azurophil granule proteins and it also plays an important role in neutrophil function during inflammatory processes, including degradation of extracellular matrix components and cytokines, modulation of integrin clustering on neutrophils and direct chemoattraction of T

cells and other leukocytes²⁹. Besides trypsin and thrombin, proteinase-activated receptor 4 is also activated by the neutrophil derived serine protease cathepsin-G³⁰.

There is strong evidence for barrier dysfunction in IBD³¹: hyperpermeability in non-involved segments of the intestine of CD patients as well as in first degree relatives has been reported³²⁻³⁴ and increased permeability have also been associated with an increased risk of relapse^{35,36}. Data are however less abundant on paracellular permeability regarding UC. Increase in gut permeability has previously been reported in clinically active UC, which was also shown to correlate with disease severity^{31,37,38}. Still, gut permeability has not yet been evaluated in remission of the disease.

AIMS

The studies were conducted (i) to investigate the origin of elevated fecal serine protease activity in IBS-D patients, (ii) to evaluate if this elevated colonic luminal serine protease activity may be sufficient to trigger alterations in colonic permeability and sensitivity in mice, (iii) to examine the possible involvement of PAR-2 activation in this process and (iv) to analyze the underlying molecular mechanisms in the tight junction. We also aimed to (v) evaluate whether the high colonic luminal serine-protease activity in UC may contribute to increased gut permeability and (vi) to examine the possible involvement of cathepsin-G and PAR-4 in this process. Furthermore, we aimed (vii) to measure intestinal and colonic permeability of patients with IBS of the diarrhea and of the constipation-predominant subtype in vivo, (viii) to investigate possible correlation between increased gut permeability and clinical symptoms in IBS-D patients and (ix) to measure gut permeability in patients with ulcerative colitis in remission.

PATIENTS AND METHODS

1. Patient selection

1.1. Fecal serine proteases in IBS and in UC, their role in mediating increased colonic permeability and sensitivity in murine model

Demographic data of the patients enrolled in the study are summarized in Table 1. Patients fulfilling the Rome II criteria for IBS (patient screening was carried out in 2005-2006) participated in the study³⁹. All participants provided medical history and underwent physical examination. In IBS patients other gastrointestinal disorders were excluded by detailed blood and stool analyses, serological assays for coeliac disease, lactose–hydrogen breath test and colonoscopy. Active UC was assessed clinically and endoscopically. The study protocol was approved by the Ethical Committee of the University of Szeged. All subjects provided written and informed consent to participate.

| Group | Number of patients | Age mean (range) | Sex ratio (M/F) |
|------------------|--------------------|------------------|-----------------|
| IBS-D | 24 | 49 (19-75) | 9/42 |
| IBS-C | 18 | | |
| IBS-A | 10 | | |
| UC | 17 | 41 (18-79) | 6/11 |
| INF | 23 | 52 (19-90) | 12/11 |
| Healthy subjects | 25 | 44 (30-65) | 4/21 |

Table 1. Demographic data. IBS-D: diarrhea-predominant IBS patients, IBS-C: constipation-predominant IBS patients, IBS-A: IBS patients with altered bowel habit, UC: patients with ulcerative colitis, INF: patients with acute infectious diarrhea.

1.2. In vivo gut permeability in IBS and in inactive ulcerative colitis

Demographic data of the patients enrolled in the study are summarized in Table 2. Thirty patients fulfilling the Rome III criteria for IBS participated in the study, none of which related the onset of their symptoms to infectious gastroenteritis. According to the Rome III criteria introduced in 2006, symptoms are expected to originate 6 months before diagnosis, which is a less restrictive timeframe compared to the Rome II criteria (12 weeks of symptoms over 12 months). In addition, according to Rome III criteria IBS subtyping should be based on stool

consistency, which results in four subgroups, namely diarrhoea predominant (IBS-D), constipation predominant (IBS-C), subjects with mixed pattern (IBS-M) and unsubtyped IBS (IBS-U). Still, bowel subtyping used in Rome II for IBS-D and IBS-C remained acceptable. Other gastrointestinal disorders were excluded by detailed blood and stool analyses, serological assays for celiac disease, lactose-hydrogen breath test and colonoscopy. Patients with inactive ulcerative colitis (partial Mayo score \pm SEM: 1.3 ± 0.2 ; CRP (mg/dl) \pm SEM: 3.8 ± 1.3) were previously shown to have either left-sided colitis or pancolitis. Voluntary subjects, free of any gastrointestinal symptoms, served as controls. Patients and voluntary subjects with impaired renal function, alcohol consumption, using NSAIDs, prokinetics, antihistamines or immunosuppressive agents were excluded from the study. UC patients were required to be exclusively on 5-ASA maintenance therapy. The study protocol was approved by the Human Investigation Review Board, University of Szeged. All subjects provided written and informed consent to participate.

| Group | Number of patients | Age mean (range) | Sex ratio (M/F) |
|------------------|--------------------|------------------|-----------------|
| IBS-D | 18 | 49 (25-68) | 6/12 |
| IBS-C | 12 | 56 (37-65) | 2/10 |
| UC | 13 | 47 (29-72) | 3/10 |
| Healthy subjects | 10 | 49 (38-65) | 2/8 |

Table 2. Demographic data

2. Methods

2.1. Fecal samples

Only feces collected in situ, or collected at home and transported within 1 h after defecation to the First Department of Internal Medicine in Szeged, Hungary, were used. Samples were stored at -80°C until transportation on dry ice to INRA Toulouse, France. Upon arrival, 1g of fecal sample was thawed, dissolved, and homogenised in 7ml of Tris buffer, centrifuged (4500 rpm, 10 min, $+4^{\circ}\text{C}$) and filtered ($0.2\ \mu\text{m}$, Nalgene, (Adventure 16, San Diego, California, USA)). The acquired supernatants were used for measuring total protease activity, serine protease activity, polymorphonuclear neutrophil (PMN) and pancreatic elastase,

myeloperoxidase (MPO), calprotectin and human secretory leucocyte protease inhibitor (SLPI) activity.

2.2. Measurement of fecal enzymatic activities

To measure total fecal serine protease activity, supernatants of fecal homogenates (25 ml) were incubated with 1 ml of reaction buffer (0.15 M NaCl and 20 mM Tris-HCl, pH 8.3) and 1ml of 0.5% (w/v) azocasein (Sigma, St Quentin Fallavier, France) at 40°C. The reaction was stopped after 20 minutes with 1ml of 10% (v/v) trichloroacetic acid (TCA, Sigma). Following centrifugation, absorption of the clear supernatant was measured at 366 nm. Enzymatic activities of the supernatants were normalized to protein content. To determine whether protease activity was dependent upon serine proteases, measurements were done after preincubation for 30 min with selective serine protease inhibitors, AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride) and a mixture of soybean trypsin inhibitor (SBTI) and aprotinin (Sigma). Protease activity was expressed as units per milligram of protein, standardized against activity elicited by 1 U of standard trypsin. Pancreatic elastase-1, SLPI, PMN elastase and calprotectin concentrations were assayed by ELISA (Schebo-Tech, Giessen, Germany; R& D Systems, Lille, France; Immundiagnostik AG, Bensheim, Germany; HyCult Biotechnology, Uden, The Netherlands, respectively). Fecal MPO activity was measured as described earlier⁴⁰. Cat-G activity was measured in fecal supernatants from patients with UC and healthy subjects, using N-succinyl-Ala-Ala-Pro-Phe p Nitroanilide (Sigma) as a substrate. Enzymatic activity was measured at 410 nm for 5 minutes at 37°C.

2.3. Animals

Congenic 6–9-week old male C57BL/6J wild type (Janvier, Le Genest St-Isle, France) and PAR-2-deficient mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. The genetic status of the PAR-2-deficient mice was confirmed by PCR. Mice were housed in polypropylene cages in a light- and temperature-controlled room (12 h/12 h cycles; 20±2°C), were fed standard pellets (Harlan Teklad, Bicester, Oxon, UK), and water was provided ad libitum. The experimental protocols described in the study were approved by the local Institutional Animal Care and Use Committee.

2.4. Visceral sensitivity model

Under xylazine/ketamine anaesthesia (both 1.2 mg, subcutaneously), two nickel–chrome electrodes were implanted into the abdominal external oblique muscle and a third into the abdominal skin, and were exteriorized on the back of the neck. On the fifth to seventh postoperative day, colorectal distensions were used as noxious stimuli to evaluate visceral hyperalgesia by electromyographic (EMG) recording. Under sodium pentobarbital anaesthesia (10mg, intraperitoneally), polyethylene perfusion and distension catheters (Fogarty catheter for arterial embolectomy, 4F, Edwards Lifesciences, Nijmegen, The Netherlands) were inserted into the colon. Animals received 0.3 ml of fecal supernatants of IBS-D or IBS-C (constipation-predominant IBS) patients, of healthy subjects or of IBS-D patients previously incubated with serine protease inhibitors, SBTI and aprotinin. The colorectal distension procedure started 60 min after the infusion of the supernatants had finished with volumes progressively increasing in 0.02 ml steps, from 0 to 0.12 ml, each step lasting 10 s with 5 min non-distension periods in between. During the distension periods, the striated muscle's EMG activity was recorded and analysed according to Larsson *et al*⁴¹. Basal EMG activity was subtracted from the EMG activity registered during the periods of distension.

2.5. In vitro permeability model

Mice were sacrificed by cervical dislocation and the distal part of the colon was removed. Colonic strips were mounted with a flux area of 0.3 cm² in Easymount Ussing-type chambers (Physiologic Instruments, San Diego, California, USA), bathed in Krebs solution and oxygenated at a maintained temperature of 37°C. After allowing 15 min for equilibrium, one-fifth of the initial volume of the buffer solution (1ml) of the apical compartment (mucosal side) was replaced with physiological saline or supernatants (500ul) and fluorescein isothiocyanate (FITC)-labelled dextran (500ul) (4000 Da, 0.022 g/ml, Sigma). 60 minutes later fluorescent intensity was measured on the serosal side of the chamber. Supernatants derived from healthy subjects, from IBS patients or from UC patients. IBS-D supernatants were alternatively pre-incubated with protease-inhibitors soybean trypsin inhibitor (SBTI) and aprotinin for 30 minutes on ice and then added to the mucosal side of the colonic strip. To

assess the potential role of PAR-1, PAR-2 and PAR-4 in the effect of UC fecal supernatants on colonic paracellular permeability, selective receptor antagonists were used: PAR1 antagonist FLLRN (Phe-Leu-Leu-Arg-Asn, 10 μ mol/L, Peptides International, Louisville, KY)^{42,43} (3/13,14), PAR2 antagonist FSLLRY (Phe-Ser-Leu-Arg-Tyr, 10 μ mol/L, Bachem, Weil am Rhein, Germany)⁴⁴ and P4-pal10 pepducin (final concentration 1 μ mol/L) (NeoMPS, Strasbourg, France) were added to the mucosal side of the chamber prior to the administration of fecal supernatants to the mucosal side. The effect of the PAR-4 agonist peptide (Ala-Tyr-Pro-Gly-Lys-Phe-NH₂, 50 μ mol/L, Sigma) was also assessed, as well as the effect of a selective Cathepsin-G inhibitor (SCGI): UC fecal supernatants were pre-incubated for 30 minutes on ice with the inhibitor (0.2mmol/L) and then the mixture was added to the mucosal side of the colonic strip.

2.6. Immunohistochemistry of pMLC and ZO-1

At 1 and 4 h after the intracolonic infusion of supernatants of IBS-D patients or healthy controls, saline, SLIGRL (5 mg) or IBS-D supernatants previously incubated with serine protease inhibitors, the mice were sacrificed and the distal colon was removed. For both phosphorylated MLC (pMLC) and ZO-1 immunolabelling, samples were fixed in buffered paraformaldehyde (4%), incubated in 30% sucrose (24 h, +4°C), embedded (Tissue Tek medium) and frozen in isopentane at -45°C. Cryostat sections (7 μ m) were fixed with acetone (10min, -20°C), hydrated in phosphate-buffered saline (PBS) and treated with 4 mg/ml sodium borohydride (45min, +4°C). Sections were permeabilized with PBS-0.5% Triton X-100 and incubated in blocking solution (PBS containing 1% bovine serum albumin). For pMLC staining, samples were then incubated with goat anti-pMLC antibodies (1/100, SantaCruz, Santa Cruz, California, USA) followed by incubation with biotin-conjugated IgG donkey anti-goat antibody (1/1000, Interchim, Montlucon, France). Sections were rinsed in NaHCO₃ (0.1M, pH 8.2) and incubated with FITC-conjugated avidin (1/500) diluted in the same solution. For ZO-1 labelling, sections were incubated with rabbit anti-ZO-1 antibodies (1/500, Zymed, San Francisco, California, USA) followed by incubation with Alexa fluor 488-conjugated IgG donkey anti-rabbit antibodies (1/2000, Molecular Probes, Cergy-Pontoise, France). All sections were mounted in Vectashield HardSet Mounting Medium with

4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California, USA) and examined under a Nikon 90i fluorescent microscope.

2.7. Western blot for pMLC

Colonic mucosa was collected from mice 1h after intracolonic infusion with fecal supernatants of healthy subjects or IBS-D patients, or saline. Proteins were extracted with RIPA buffer and quantified. Following the Laemmli method⁴⁵, equal amounts of protein extracts were electrophoresed by 12% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and then electrotransferred onto Hybond-P membrane (GE Healthcare, Bordeaux, France). After saturation, the membrane was incubated with anti-pMLC primary antibody (1/1000, Biosource, Worcester, Massachusetts, USA) and peroxidase-conjugated goat anti-rabbit secondary antibody (1/1000, Millipore, St Quentin en Yvelines, France). The membrane was developed with SuperSignal Reagent (Pierce, Prebieres, France). Integrated density values were assessed by ImageJ 1.37 software (NIH, Bethesda, Maryland, USA).

2.8. In vivo permeability measurement with ⁵¹Cr-EDTA

To measure intestinal and colonic permeability after an overnight of fasting participants emptied their bladders and consumed ⁵¹Cr-EDTA (Perkin Elmer Life Sciences, Boston, MA, USA) of 1.8 MBq activity dissolved in 100 ml of water, followed by 200 ml of standard meal (Nutridrink, Nutricia, Budapest, Hungary) containing 300 kcal. Study participants were requested to restrain from drinking for 3 hours and from eating for 5 hours. Gut permeability was evaluated by measuring 24-hour urine excretion of orally administered ⁵¹Cr-EDTA, where time periods were chosen to relate to permeability within the proximal (0-3h) and distal (3-5h) small intestine and the large bowel (5-24h)^{38,46,47}. Urinary output was recorded for each period and the radioactivity of 1 ml aliquots were counted by a gamma-counter (Packard Cobra, Canberra Packard, UK) in duplicates. Gut permeability was expressed as percentage of urinary excretion of the orally administered dose of ⁵¹Cr-EDTA (%).

2.9. Evaluation of symptoms in IBS-D patients

IBS-D patients were asked to fill out a questionnaire, regarding their clinical symptoms at the time of the permeability measurement. Stool frequency (/week) and consistency (according to the Bristol stool scale, which evaluates stool consistency according to a visual scale graded 1: very hard-7: watery), frequency of abdominal pain, distension and bloating (/week), intensity of abdominal pain, distension and bloating and quality of life (visual analogue scale /VAS/; %) were the symptoms being evaluated and correlated to colonic permeability.

3. Statistical analysis

All data are presented as means \pm SEM. For statistical analysis, Prism 4.0 (GraphPad, San Diego, California, USA) was used. Multiple comparisons for fecal enzymatic activities of different patient groups and integrated optical density values of pMLC western blots were analyzed by repeated measures of one-way analysis of variance (ANOVA), followed by Tukey's posttest or Kruskal–Wallis posttest (for MPO activity). Statistical significance for visceral hypersensitivity results was established by using two-way ANOVA, followed by Bonferroni posttest. In vitro permeability results were analyzed with one-way ANOVA, followed by Tukey posttest or two-tailed unpaired t test. Multiple comparisons for in vivo permeability of different patient groups were analyzed by repeated measures of one-way ANOVA, followed by Tukey's posttest. Unpaired t-test was used to evaluate colonic permeability data in subgroups of UC patients. Linear regression was applied to establish correlation between clinical symptoms and permeability. Statistical significance was accepted at $p < 0.05$.

RESULTS

1. Fecal serine protease activity

In healthy subjects the total fecal serine protease activity was 698 U/mg of protein. This serine protease activity was significantly greater in IBS-D patients (2079 U/mg, $p < 0.001$) and in UC patients (2193 U/mg, $p < 0.01$) compared with healthy controls; a similar increase was

not present regarding the IBS-C, IBS-A subgroups or patients with acute infectious diarrhea (INF) (Figure 4A). Addition of the serine protease inhibitor AEBSF abolished this increased protease activity in IBS-D and UC supernatants. A similar inhibition was obtained with preincubation with SBTI and aprotinin, two common serine protease inhibitors validated for human use (Figure 4B). Regarding the potential origin of the elevated serine protease activity, no significant difference was observed in fecal pancreatic elastase-1 concentration in UC or in any subgroups of IBS patients when compared with controls (Figure 4C). However, pancreatic elastase-1 concentration was significantly decreased in INF patients ($p < 0.05$), which might be attributed to the diluted fecal content (i.e. profuse watery diarrhea) and restricted diet. PMN-derived elastase had a significantly elevated fecal concentration in UC and INF patients ($p < 0.05$), but not in any subgroup of IBS patients (Figure 4D). Human fecal SLPI activity showed no significant difference in any of the studied groups of patients when comparing them with control subjects (Figure 4E). Fecal inflammatory markers, such as human calprotectin and MPO, showed a significant increase in UC ($p < 0.001$ and $p < 0.05$, respectively) and INF patients ($p < 0.05$ and $p < 0.001$, respectively), but we found no increase in any of the IBS subgroups (Figure 4F and G).

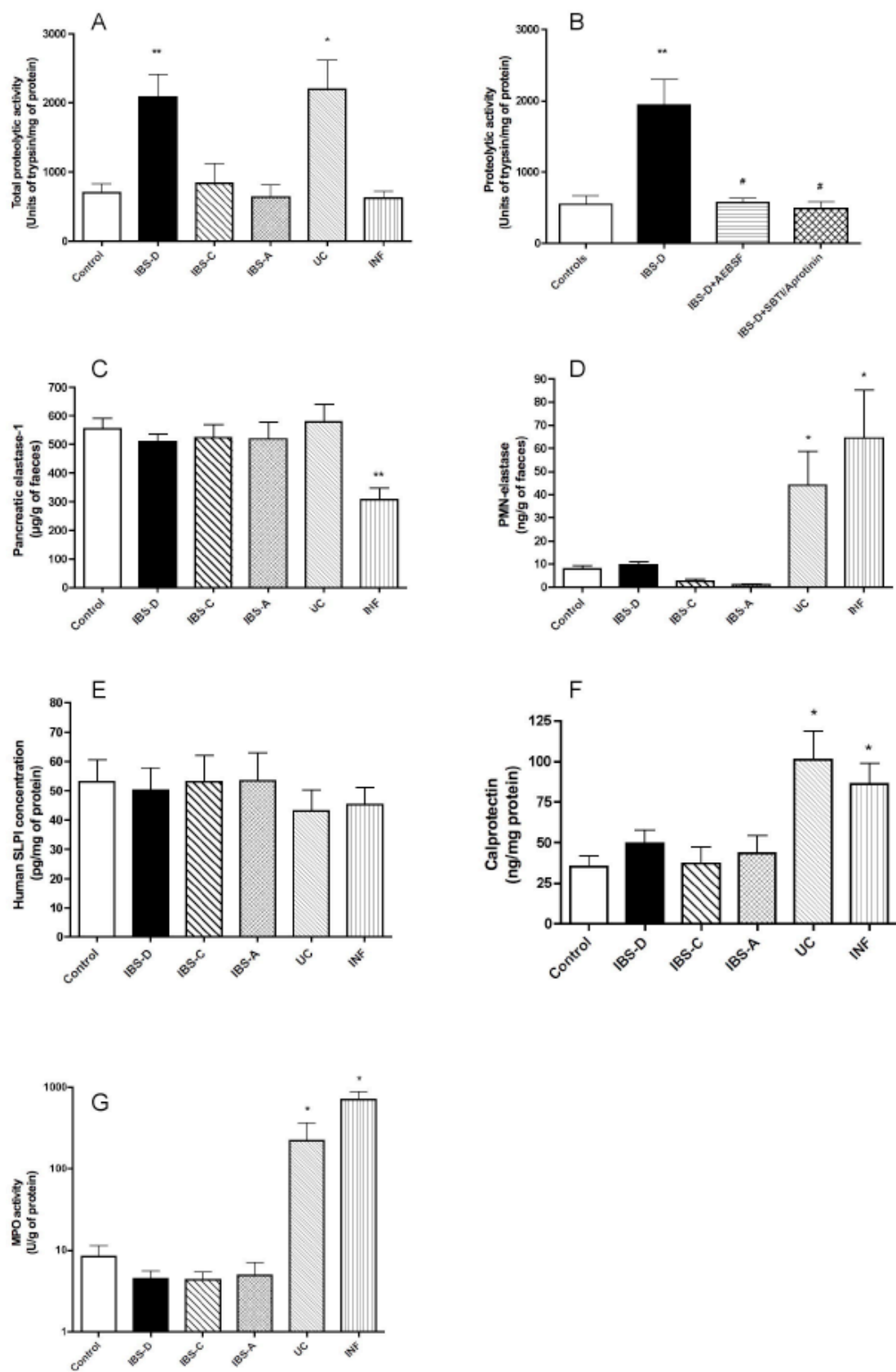


Figure 4.

Figure 4. The increase in total fecal serine protease activity in diarrhea-predominant irritable bowel syndrome (IBS-D) is neither accompanied by elevated enzymatic activity of pancreatic or inflammatory cell origin nor coupled with decreased antiprotease activity. **(A)** Fecal total protease activity in healthy controls, in IBS-D patients, IBS-C(constipation-predominant) patients, those with alternating bowel habits (IBS-A), patients with active ulcerative colitis (UC) and those with acute infectious diarrhea (INF). **(B)** Fecal protease activity after incubation with serine protease inhibitors AEBSF(4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride), or a mixture of soybean trypsin inhibitor and aprotinin. **(C)** Fecal pancreatic elastase-1 concentration. **(D)** Fecal polymorphonuclear neutrophil (PMN) -elastase concentration. **(E)** Fecal human secretory leucocyte protease inhibitor (SLPI) concentration. **(F)** Fecal calprotectin concentration. **(G)** Fecal myeloperoxidase (MPO) activity. * $p < 0.05$, ** $p < 0.01$, compared with healthy controls, # $p < 0.05$, compared with IBS-D (one-way analysis of variance). Error bars represent SEM.

2. Visceral hypersensitivity is triggered by elevated serine protease activity of IBS-D supernatants and is dependent on mucosal PAR-2 expression

Intracolonic infusion of fecal supernatants from IBS-D patients administered prior to colorectal distensions in mice significantly increased the abdominal muscle EMG response (a valid criterion representing nociception, Figure 5A) at low distension volumes, namely at 0.02, 0.04 and 0.06 ml, compared with the intensity of muscle contractions in animals treated with fecal supernatants of control subjects (0.02 ml, 30.6 (5.9) mV/s vs 3.5 (1.7) mV/s; 0.04 ml, 73.4 (7.9) mV/s vs 18.9 (7.5) mV/s; 0.06 ml, 107.4 (4.4) mV/s vs 53.2 (14.3) mV/s; $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively, Figure 5B). Conversely, colonic instillation of IBS-C supernatants did not evoke alteration of visceral sensitivity to colorectal distension (Figure 5C). Incubation of fecal supernatants from IBS-D patients with serine protease inhibitors prior to colonic infusion, prevented the increased EMG response to low volumes of distension (0.02 ml, 2.8 (1.4)mV/s, 0.04 ml, 49.8 (2.9) mV/s; $p < 0.01$, $p < 0.05$, respectively, Figure 5B), while protease inhibitors per se had no effect (data not shown). Furthermore, IBS-D supernatants failed to induce allodynia or visceral hypersensitivity in PAR-2-deficient mice (0.02 ml, 4.6 (3.5) mV/s; 0.04 ml, 41.4 (7.0) mV/s; 0.06 ml, 59.4 (14.2) mV/s, Figure 5D).

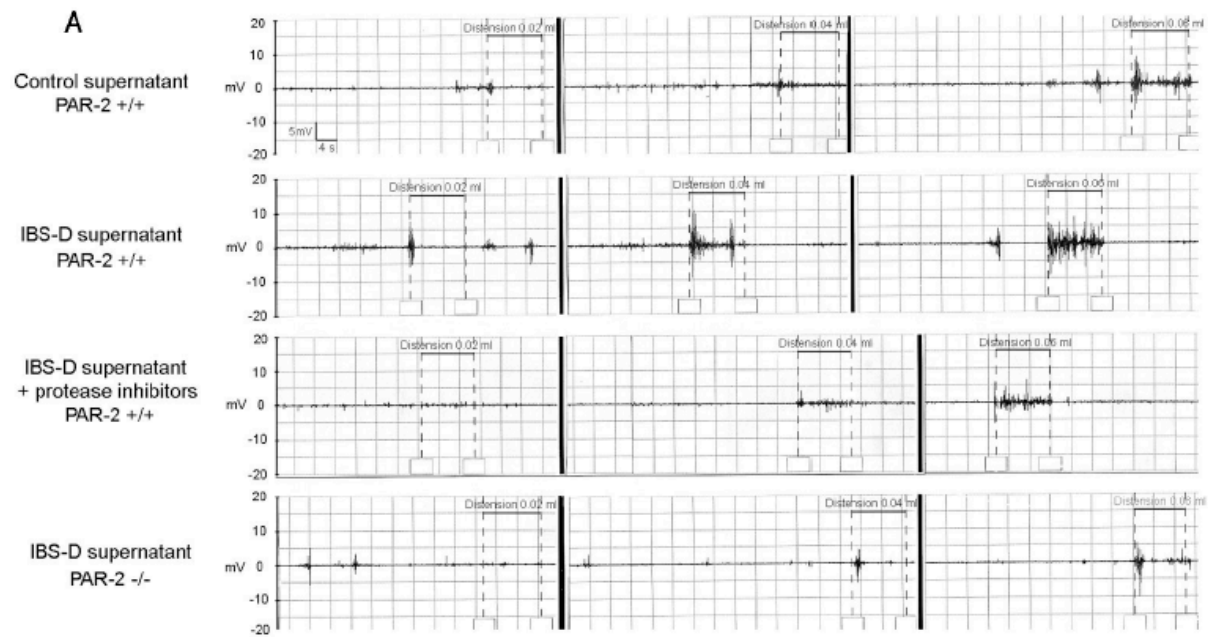


Figure 5A. Representative electromyographic recordings of abdominal muscle contractions (as a valid criterion for visceral nociception) evoked by colorectal balloon distension 60min after intracolonic infusion of fecal supernatants from diarrhea-predominant irritable bowel syndrome (IBS-D) patients (n=5), control subjects (n=5) and IBS-D supernatants previously incubated with serine protease inhibitors (n=3) in wild type or PAR-2 deficient mice (n=5) (each supernatant was tested on an average of five wild type mice).

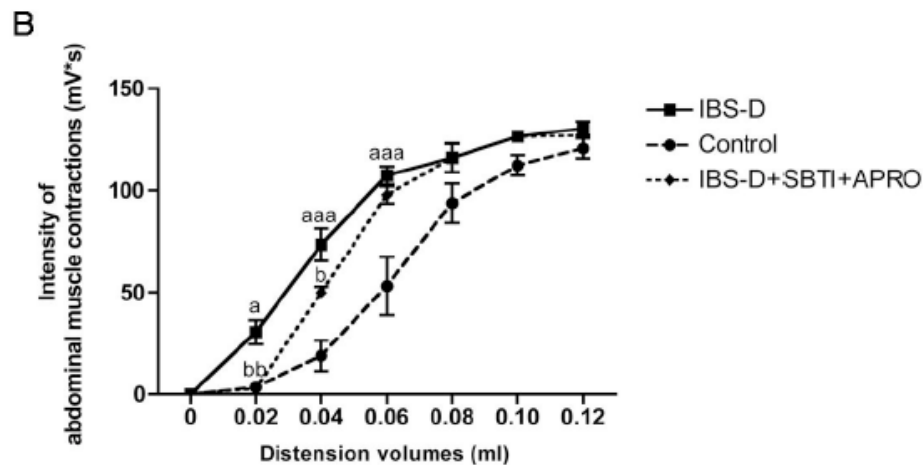


Figure 5B. Supernatants of IBS-D patients induced allodynia and visceral hypersensitivity in wild-type mice at low distension volumes compared with control supernatants (0.02, 0.04, 0.06 ml; a p <0.05, aaa p <0.001, aaa p <0.001, respectively), an effect that was partially prevented by the administration of serine protease inhibitors (at 0.02 and 0.04 ml distension volumes, bb p <0.01 and b p <0.05, respectively). APRO, aprotinin; SBTI, soybean trypsin inhibitor.

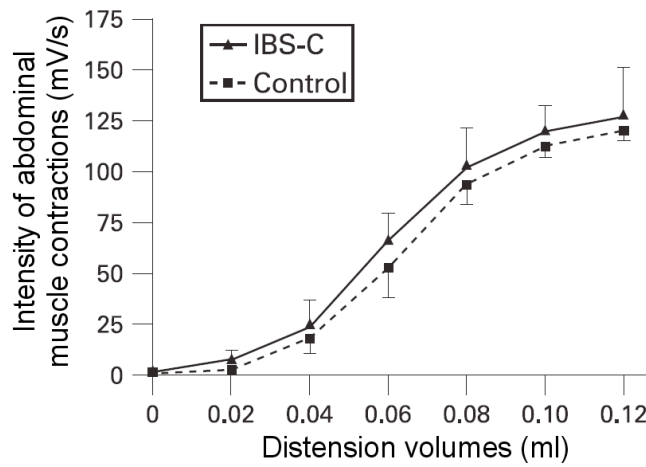


Figure 5C. Constipation-predominant irritable bowel syndrome (IBS-C) supernatants were devoid of any effect on visceral sensitivity.

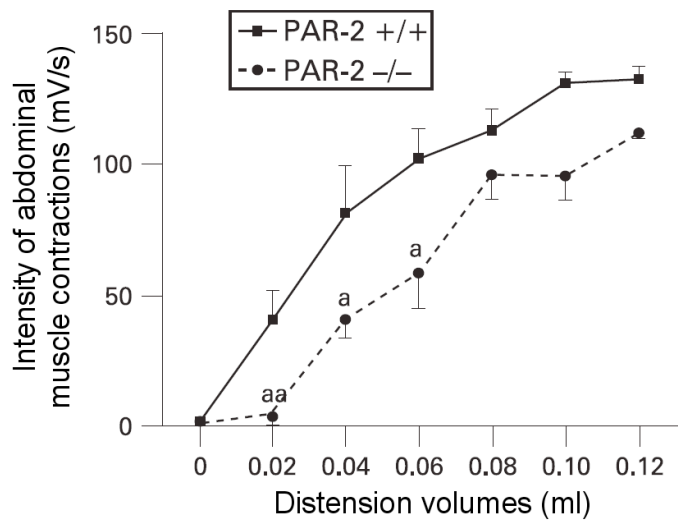


Figure 5D. IBS-D supernatants did not trigger visceral hyperalgesia in PAR-2-deficient mice (0.02, 0.04, 0.06ml; ^{aa} $p < 0.01$, ^a $p < 0.05$, ^a $p < 0.05$, respectively).

3. Increase in colonic paracellular permeability (CPP) is evoked by elevated serine protease activity of IBS-D fecal supernatants and is PAR-2 dependent

Administration of fecal supernatants from healthy subjects to the mucosal side of the murine colon mounted in Ussing chambers did not significantly alter CPP compared with saline (128% vs. 100%; ns.). In contrast, addition of fecal supernatants from IBS-D patients significantly increased the FITC–dextran flux compared with the administration of supernatants from healthy subjects or saline (192%, $p < 0.05$). Comparatively, supernatants from IBS-C patients failed to induce such an increase in colonic permeability (137%; ns., Figure 6A). Previous incubation of the supernatants from IBS-D patients with SBTI and aprotinin prior to administration significantly decreased the elevated CPP evoked by supernatants from IBS-D patients (111% vs. 202%, $p < 0.01$, Figure 6B). No increase in CPP was observed in response to fecal supernatants from IBS-D patients on colonic strips of PAR-2-deficient mice in contrast to their wild-type strain (127% vs. 248%, $p < 0.05$, Figure 6C).

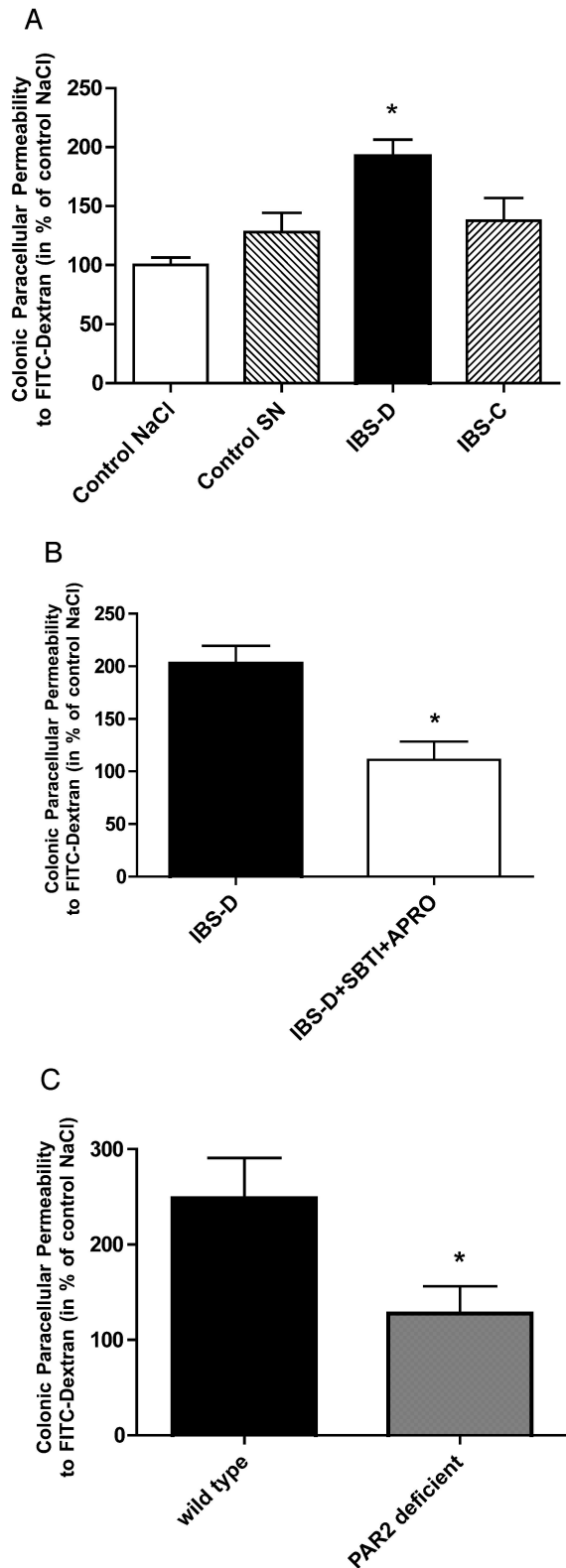


Figure 6.

(A) Colonic paracellular permeability (CPP) to fluorescein isothiocyanate (FITC)-dextran in Ussing chambers 60 min after the administration of fecal supernatants of diarrhea-predominant irritable bowel syndrome (IBS-D) patients, constipation-predominant IBS (IBS-C) patients and healthy controls on colonic strips of wild-type mice (* $p < 0.05$ compared with control supernatant).

(B) CPP assayed after prior incubation of IBS-D supernatants with serine protease inhibitors (* $p < 0.01$).

(C) CPP in wild-type and proteinase-activated receptor-2 (PAR-2)-deficient mice 60 min after the administration of supernatants from IBS-D patients (* $p < 0.05$). CPP evoked by supernatants was expressed as percentage of CPP induced by their saline controls.

4. Increased rapid phosphorylation of MLC and delayed redistribution of ZO-1 in epithelial cells after mucosal exposure to IBS-D fecal supernatants

One hour after intracolonic infusion with supernatants from IBS-D patients there was a pronounced and diffuse labelling of pMLC in epithelial cells, similar to that observed after the infusion of SLIGRL used as a positive control for PAR-2 activation, and inhibited by preincubation with serine protease inhibitors. Administration of supernatants from healthy subjects or saline failed to evoke an increase in pMLC immunostaining, which remained restricted to the tight junction area (Figure 7A).

Four hours after intracolonic infusion of supernatants from healthy subjects or saline, selective immunostaining of the tight junction protein ZO-1 showed labelling that was restricted to the surface of epithelial cells. In contrast, intracolonic infusion of supernatants from IBS-D patients resulted in a marked labelling of the intracellular compartment, similarly to that experienced after the infusion of SLIGRL, suggesting intensive internalisation of the protein. Previous incubation of fecal supernatants from IBS-D patients with serine protease inhibitors prevented the occurrence of a marked intracellular labelling of colonocytes (Figure 7B). Western blotting showed an increased level of pMLC in the colonic mucosa of mice 1 h after the intracolonic infusion of supernatants from IBS-D patients compared with those infused with supernatants from healthy controls ($p < 0.001$). There was no significant difference in the level of pMLC in the colonic mucosa of mice that were infused with supernatants from healthy subjects or IBS-D supernatants previously incubated with a mixture of serine protease inhibitors (Figure 7C).

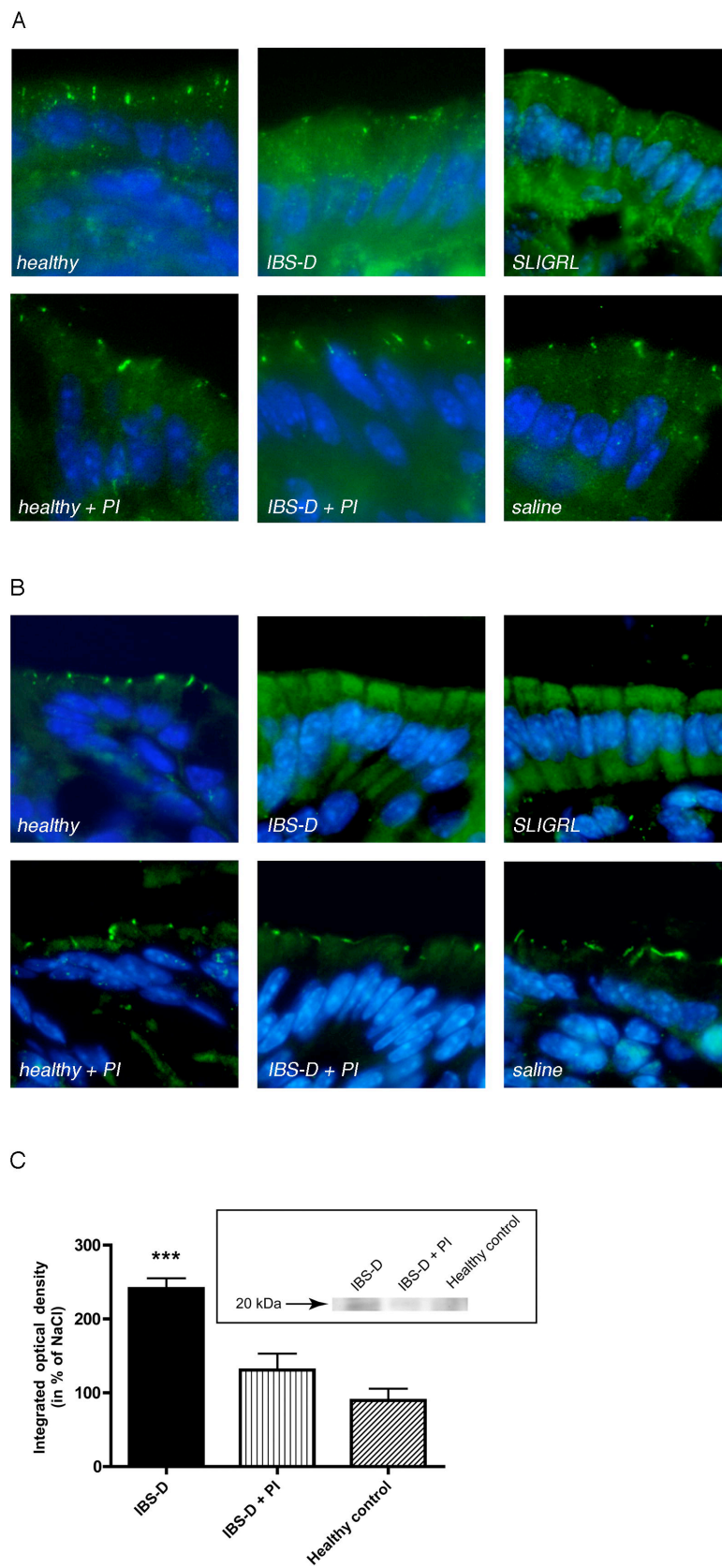


Figure 7.

Figure 7. (A) Immunolabelling of phosphorylated myosin light chain (pMLC, green) and nuclear labelling with 4,6-diamidino-2-phenylindole (DAPI, blue) in enterocytes 1 h after intracolonic infusion with supernatants from healthy controls, diarrhea-predominant irritable bowel syndrome (IBS-D) patients, SLIGRL, control and IBS-D supernatants previously incubated with serine protease inhibitors and saline. **(B)** Immunostaining of zonula occludens-1 (ZO-1, green) and nuclear labelling with DAPI (blue) in enterocytes 4 h after intracolonic infusion with supernatants from healthy controls, IBS-D patients, SLIGRL, control and IBS-D supernatants previously incubated with serine protease inhibitors and saline. **(C)** Western blotting of pMLC in mice colonic mucosa after intracolonic infusion with supernatants from IBS-D patients, healthy controls and IBS-D supernatants previously incubated with serine protease inhibitors. pMLC bands can be identified at 20 kDa. Quantitative analysis of the blots showed a significantly higher level of pMLC in colonic mucosa infused with IBS-D supernatants compared with healthy controls, *** $p < 0.001$ (PI: protease inhibitors).

5. Increase in colonic paracellular permeability (CPP) is evoked by cathepsin-G of UC fecal supernatants and is PAR-4 mediated

Administration of fecal supernatants from healthy subjects to the mucosal side of murine colon mounted in Ussing chambers did not significantly alter CPP, as compared with saline (1.58 ± 0.29 vs. 2.00 ± 0.28 nmol/h/cm², ns., Figure 8A). In contrast, addition of fecal supernatants from UC patients significantly increased the FITC-dextran flux compared with supernatants from healthy subjects (3.34 ± 0.47 nmol/h/cm²; $p < 0.05$) and saline ($p < 0.01$) (Figure 8A). The addition of PAR-1 antagonist FLLRN (10 μ mol/L) and PAR-2 antagonist FSLLRY (10 μ mol/L) did not significantly alter the increased CPP triggered by UC fecal supernatants (Figure 8B). In contrast, pepducin P4pal-10 significantly reduced the effect of UC fecal supernatants on permeability (1.84 ± 0.20 nmol/h/cm²; $p < 0.05$, Figure 8A). Finally, PAR4 activating peptide (AYPGKFNH₂; 50 μ mol/L) increased the dextran flux across mice colonic epithelium by 199% in comparison with vehicle (2.69 ± 0.17 vs. 0.90 ± 0.17 nmol/h/cm², $p < 0.001$, Figure 8C). Fecal supernatant from patients with UC showed high activity, whereas those from healthy subjects presented weak ability to cleave the substrate N-succinyl-Ala-Ala-Pro-Phe p-Nitroanilide, which is widely used to assess Cat-G activity (28.34 ± 3.05 vs. 2.02 ± 0.22 , $p < 0.001$, Figure 9A). Moreover, SCGI decreased enzymatic activity in UC supernatants by 43% (16.14 ± 2.02 , $p < 0.01$, Figure 9A), showing that Cat-G is present in significant amounts within the lumen in UC, but not in healthy subjects. Preincubation of UC fecal supernatants with SCGI abolished the effect of UC fecal supernatants by 77% on paracellular permeability (3.49 ± 0.53 vs. 2.01 ± 0.30 , $p < 0.05$, Figure 9B).

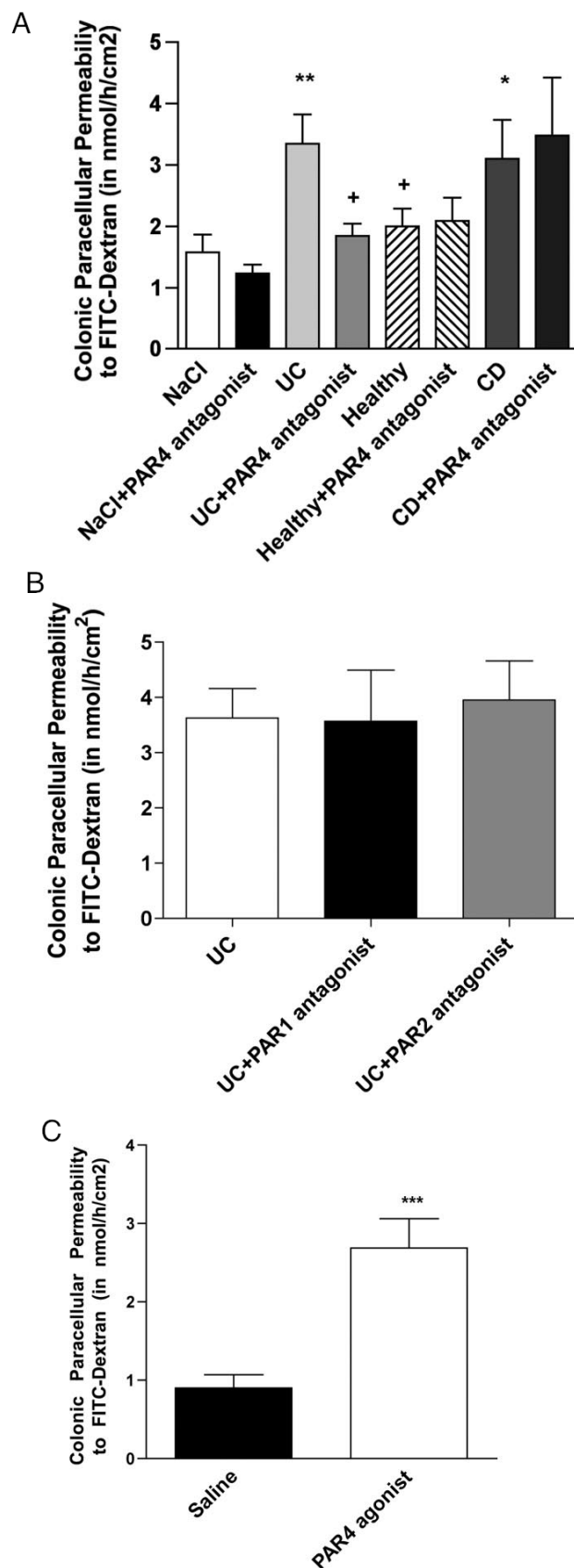


Figure 8.

(A) Colonic paracellular permeability (CPP) to FITC-Dextran in Ussing chambers 60 minutes after the administration of fecal supernatants of UC patients (n =7), healthy controls (n=6), and saline (n=10) on colonic strips of wild-type mice, and CPP after prior incubation of fecal supernatant of UC patients (n =15), healthy controls (n=12), and saline (n=12) with pepducin P4pal-10 targeting PAR4 (N-palmitoyl-SGRRYGHALR-NH₂, 1μmol/L). +p<0.05 compared with fecal supernatants of UC patients, *p<0.05 compared with saline and **p<0.01 compared with saline. (B) CPP after prior incubation of fecal supernatant of UC patients (n=6) with the PAR1 antagonist, FLLRN (10μmol/L) and the PAR2 antagonist, FSLLRY-amide (10μmol/L). No significant differences were observed. (C) CPP 60 minutes after the administration of PAR4 agonist (AY-NH₂, 50μmol/L) (n =10) and saline controls (n=7) on colonic strips of wild-type mice. Data are expressed as means SEM, ***p<0.001.

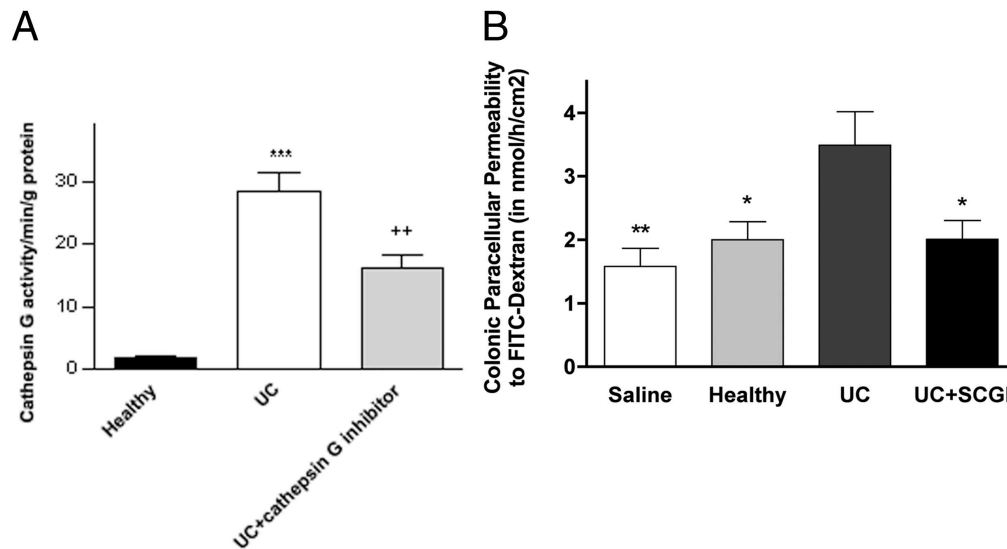


Figure 9.

(A) Cat-G activity assessed with the substrate N-Suc-Ala-Ala-Pro-Phe-pNA, in fecal supernatants from healthy subjects (n=6) and UC patients (n=9), and UC supernatants pretreated with a specific Cat-G inhibitor (SCGI) (1 μ mol/L). Data are expressed as means \pm SEM, ***p<0.001 compared with fecal supernatants from healthy subjects, and **p<0.01 compared with fecal supernatants from UC patients. **(B)** Colonic paracellular permeability to FITC-Dextran after prior incubation of fecal supernatants from UC patients with SCGI (0.2 μ mol/L). Data are expressed as means \pm SEM (n=10), *p<0.05, **p<0.01, compared with fecal supernatants from UC patients.

6. In vivo intestinal and colonic permeability in IBS and in inactive UC patients

Twenty-four hours urinary excretion of orally administered ^{51}Cr -EDTA showed significant increase in the IBS-D and UC groups of patients compared to control subjects (3.93 ± 0.43 and 5.39 ± 0.61 vs. $1.97 \pm 0.33\%$, p<0.05 and p<0.001 respectively). Gut permeability in IBS-C patients remained as low as those of controls showing no significant difference ($1.34 \pm 0.2\%$) (Figure 10A).

Results were consistent with the above when time periods were chosen to relate to permeability within the proximal (0-3h) and distal (3-5h) small intestine and the large bowel (5-24h) during twenty-four-hour urine excretion of orally administered ^{51}Cr -EDTA. There was no significant difference in the proximal small intestinal permeability in IBS-D and inactive UC patients compared to controls (0.63 ± 0.08 and 0.82 ± 0.09 vs. $0.63 \pm 0.1\%$, respectively). However, proximal small intestinal permeability of IBS-C patients was significantly

decreased compared to controls ($0.26 \pm 0.05\%$; $p < 0.05$, Figure 10B). Gut permeability did not show any significant difference regarding the distal small intestine in the diarrhea- and constipation-predominant subgroups of IBS patients, and patients with inactive UC compared to control subjects (0.61 ± 0.12 , 0.39 ± 0.08 , 0.83 ± 0.09 vs. $0.43 \pm 0.07\%$, respectively, Figure 10C). Colonic permeability of IBS-C patients remained as low ($0.69 \pm 0.12\%$), as those of control subjects, showing no significant difference. On the contrary, colonic permeability of IBS-D patients proved to be significantly higher compared to healthy controls (2.68 ± 0.35 vs. $1.04 \pm 0.18\%$; $p < 0.05$). Furthermore, colonic permeability of patients with inactive UC was also found to be significantly elevated compared to control subjects (3.74 ± 0.49 vs. $1.04 \pm 0.18\%$; $p < 0.001$, Figure 10D). There was no significant difference in colonic permeability between patients with previous endoscopic diagnosis of left-sided colitis or pancolitis (3.26 ± 0.43 vs. $4.31 \pm 0.94\%$, ns., Figure 10E).

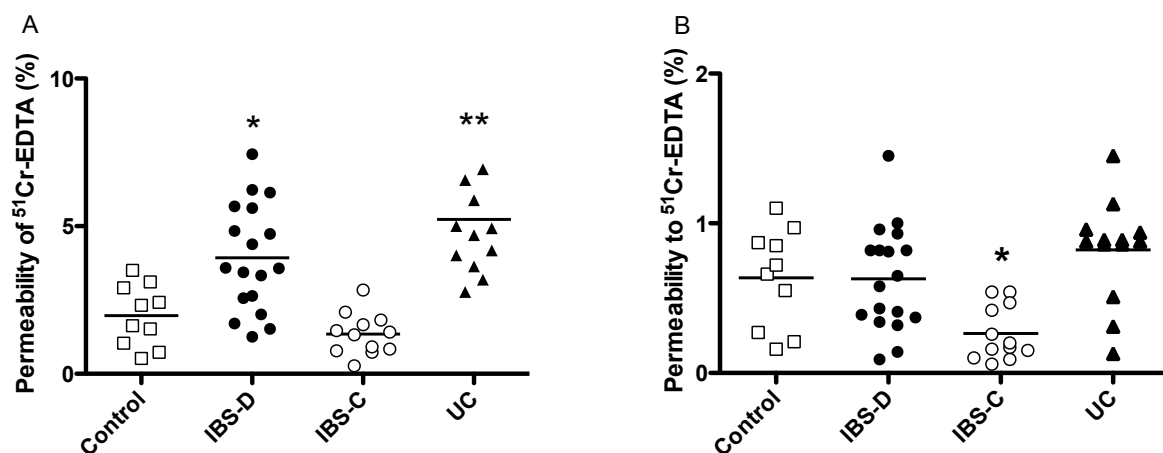


Figure 10. (A) Twenty-four-hour excretion of $^{51}\text{Cr-EDTA}$ in subgroups of IBS and inactive UC patients compared to control subjects. (B) Excretion of $^{51}\text{Cr-EDTA}$ measured between 0-3 hours after ingestion in subgroups of IBS and inactive UC patients compared to control subjects, which represents proximal small intestinal permeability.

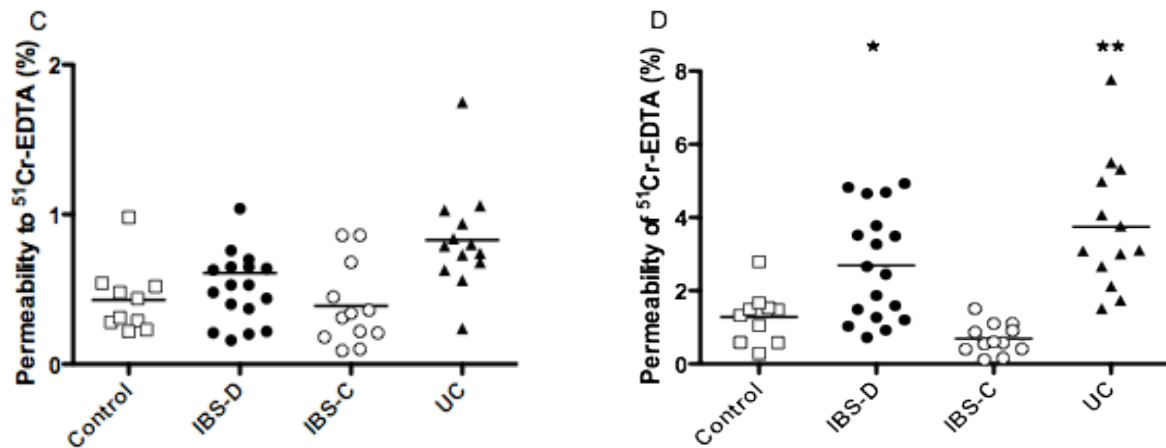


Figure 10. (C) Excretion of $^{51}\text{Cr-EDTA}$ measured between 3-5 hours after ingestion in subgroups of IBS and inactive UC patients compared to control subjects, which represents distal small intestinal permeability. (D) Excretion of $^{51}\text{Cr-EDTA}$ measured between 5-24 hours after ingestion in subgroups of IBS and inactive UC patients compared to control subjects, which represents colonic permeability.

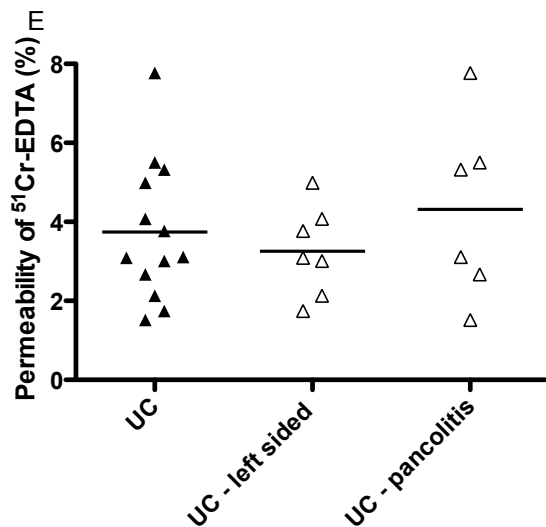


Figure 10. (E) Comparison of colonic permeability in patients with inactive left-sided colitis and pancolitis. Data are expressed as means \pm SEM, * $p < 0.05$, ** $p < 0.01$, compared with healthy controls.

7. Correlation between increased gut permeability and clinical symptoms in IBS-D patients

Stool consistency, frequency of abdominal pain, distension and bloating, intensity of abdominal pain, distension and bloating or quality of life did not show correlation with increased colonic permeability in IBS-D patients (Table 3). Nevertheless, stool frequency showed good correlation with colonic permeability in IBS-D patients ($r=0.62$; $p=0.005$, Figure 11). Colonic permeability of inactive UC patients did not show correlation with stool frequency.

| | IBS-D patients mean \pm SEM | UC patients mean \pm SEM | Correlation (Pearson r) | Correlation (p value) |
|--|----------------------------------|-------------------------------|----------------------------|--------------------------|
| Number of stools (/week) | 19.5 \pm 3.34 | | 0.62 | 0.0057 |
| Stool consistency (Bristol) | 4.94 \pm 0.26 | | -0.23 | ns. |
| Frequency of abdominal pain (/week) | 8.44 \pm 2.04 | | 0.27 | ns. |
| Intensity of abdominal pain (VAS, %) | 53.33 \pm 6.3 | | -0.22 | ns. |
| Frequency of abdominal distension (/week) | 7.83 \pm 2.03 | | 0.24 | ns. |
| Intensity of abdominal distension (VAS, %) | 56.94 \pm 5.33 | | -0.05 | ns. |
| Frequency of bloating (/week) | 5.0 \pm 0.97 | | -0.3 | ns. |
| Intensity of bloating (VAS, %) | 50 \pm 5.17 | | -0.11 | ns. |
| Quality of life (VAS, %) | 45.28 \pm 5.24 | | 0.02 | ns. |
| Number of stools (/week) | | 1.92 \pm 0.33 | -0.13 | ns. |

Table 3. Correlation between clinical symptoms and colonic permeability in IBS-D patients.

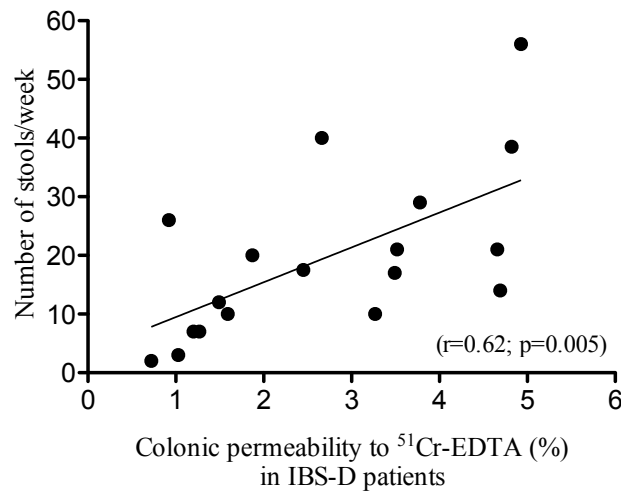


Figure 11. Stool frequency showing good correlation with colonic permeability in IBS-D patients.

DISCUSSION

In the present study we have shown that serine protease activity can be used as a marker to distinguish between IBS and acute infectious diarrhea, and the absence of increased fecal inflammatory markers permits the differentiation of IBS-D patients from UC patients. Our present investigations provide evidence that fecal supernatants from IBS-D patients are able to evoke visceral hypersensitivity in mice when applied intracolonicallly, and to increase colonic paracellular permeability in vitro. Both of these effects are prevented by the administration of serine protease inhibitors and are dependent on PAR-2 expression, since they are absent in PAR-2-deficient mice. We also showed that intracolonic infusion of supernatants from IBS-D patients promptly increases the phosphorylation of MLC, a process that is known to be involved in disrupting the integrity of epithelial tight junctions¹². Furthermore, the tight junction protein ZO-1 showed delayed internalisation in vivo in colonocytes in response to mucosal exposure to IBS-D fecal supernatants. We have also shown that fecal supernatants from UC patients are able to increase colonic paracellular permeability in vitro, an event that is induced by cathepsin-G and mediated by PAR-4. Experiments on assessing gut permeability in patients with IBS and inactive UC in vivo showed that there is no significant difference in the proximal intestinal permeability in IBS-D

and in inactive UC patients compared to controls, although in IBS-C patients a significant decrease was found. Distal small intestinal permeability is similar in all studied groups of IBS, inactive UC patients and healthy controls. Colonic permeability of IBS-C patients remains as low as those of control subjects, however colonic permeability of IBS-D patients and UC patients in remission is significantly higher than those of healthy controls. We also establish that among clinical symptoms evaluated, increased stool frequency correlates with the increase in colonic permeability in IBS-D patients.

As it has previously been shown, the increase in fecal serine protease activity is similar in patients with IBS-D and those with active UC³. Interestingly, patients with acute transient infectious diarrhea are characterised by a low level of serine protease activity, suggesting that in IBS-D patients elevated enzymatic activity is not due to an accelerated intestinal transit. In agreement with previous studies⁴⁸⁻⁵⁰, we found elevated levels of inflammatory markers in the fecal samples of UC and INF patients, but none of these markers were increased in any of the IBS subgroups. The high luminal serine protease activity in the fecal samples of IBS-D patients is neither associated with elevated enzymatic activity of pancreatic or inflammatory cell origin nor coupled with decreased antiprotease activity (SLPI), which could disturb the luminal balance between proteases and antiproteases. This is in contrast with results on elevated mast cell tryptase activity found in biopsy samples from IBS patients⁵¹. In this study an increased proteolytic activity was found in colonic washes, however its origin was not characterised, therefore we cannot exclude that the elevated activity was due to exposure to hyperosmotic laxatives used for colonoscopy preparation. In a report using fecal material and not colonic washes, no increase in mast cell tryptase was detected in fecal supernatants from IBS-D patients³. The origin of the elevated serine protease level needs further evaluation, though our data suggests microbial origin. Indeed bacteria contribute substantially to the production of colonic serine proteases⁵² and quantitative as well as qualitative alterations in fecal microbiota were described in IBS-D patients^{53,54}, namely a decrease in lactobacilli, which have high antiprotease activity. Moreover, reduced colonic microflora obtained by oral antibiotic treatment resulted in a lower serine protease activity and was associated with a decreased expression of PAR-2 on the colonic epithelial cells of mice⁵⁵. PAR-2 has been reported to be activated by pathogen proteases as well, such as *Porphyromonas gingivalis*-

produced gingipain that mediates inflammatory events in the pathogenesis of periodontitis through PAR-2⁵⁶.

Enhanced colonic sensitivity to distension in IBS patients was first demonstrated in 1973¹⁶ and since then numerous studies have confirmed altered gut sensitivity in IBS patients^{15,17,57}. In rats, PAR-2 activation with intracolonic infusion of PAR-2 activating peptide SLIGRL or trypsin provokes delayed long-lasting colorectal hypersensitivity to distension, which is associated with increased CPP¹¹. Our present results show that in a murine model allodynia is evoked by fecal supernatants of the IBS-D subgroup of patients, already by low volumes of distension. This effect was completely prevented by the administration of serine protease inhibitors at 0.02 ml, and partially at 0.04 ml. In accordance, IBS-D supernatants were not able to evoke hypersensitivity in PAR-2-deficient mice, further supporting the role of serine proteases in the initiation of visceral hypersensitivity. However, we cannot completely exclude the presence of other luminal factors besides serine proteases, which might also be involved in alterations of visceral sensitivity.

Altered intestinal permeability has been described by several clinical studies as a characteristic feature of both IBS and IBD^{13-14,18-22}. The intestinal barrier is composed of the secreted mucus layer, the structural barrier of epithelial cells and the underlying nonepithelial mucosal cells, mainly leukocytes with regulatory function⁵⁸. The main constituent of the intestinal barrier is the single layer of epithelial cells, where the paracellular space between adjacent cells is sealed by intercellular tight junctions, which represent the rate-limiting step for paracellular transit. Naturally, the barrier is severely compromised when epithelial cells are lost, as it occurs in erosions and ulcerations of active IBD, however recent data spotlight on less striking alterations, namely on altered tight junction function both in IBS¹⁸⁻²² and in IBD^{59,60}, which may serve as a structural basis for altered gut permeability. Intracolonic administration of a serine protease inhibitor, aprotinin, resulted in reduced CPP in mice⁵⁵. In agreement with this, we have shown that fecal supernatants of IBS-D patients are able to evoke increased CPP in colonic strips of mice, an effect inhibited by serine protease inhibitors, which further supports the role of serine proteases in impaired gut permeability. Accordingly, supernatants of IBS-C patients or healthy subjects, which lack elevated serine protease activity, failed to induce such permeability changes. Some studies have reported that

PAR-2 participates directly in the pathogenesis of IBS⁴. Our present data on in vitro permeability studies in PAR-2 deficient mice demonstrates that the permeability changes induced by supernatants from IBS-D patients with high serine protease activity are mediated through PAR-2. Increased intestinal permeability when triggered by intracolonic PAR-2 activation was shown to be due to MLC phosphorylation⁵¹. Our immunohistochemical studies revealed that colonic exposure to supernatants of IBS-D patients, with high protease activity, triggers a rapid phosphorylation of MLC and a subsequent, delayed internalisation of ZO-1 in colonocytes in vivo. This supports the hypothesis of a serine protease-mediated mechanism in the alteration of tight junction permeability. Our in vivo permeability measurements show that epithelial barrier dysfunction is localized to the colon and is restricted to the diarrhea-predominant subtype of IBS patients. This is in agreement with previous observations that fecal supernatants of IBS-D patients with high serine-protease activity were able to evoke immediate increase in paracellular permeability on colonic strips of mice. Therefore, we may speculate that the high concentration of serine-proteases in the colonic luminal content of IBS-D patients is also able to induce permeability changes in vivo. Our data are in contrast with a study, showing no difference in gut permeability between IBS patients and healthy controls measured by the lactulose/mannitol test and polyethylene glycols (PEGs) of different molecular weight⁶¹. Evidence shows that saccharides are degraded by colonic bacteria and PEG recovery in ileostomy patients is similar to those of healthy controls^{62,63}. Thus in contrast to ⁵¹Cr-EDTA⁶⁴, neither of these compounds can be considered ideal to measure colonic permeability, where we localized the barrier dysfunction. Our results show that ⁵¹Cr-EDTA excretion of IBS-C patients is significantly decreased in the first three hours of the experiment compared to controls, which we rather attribute to the fact that in healthy subjects ⁵¹Cr-EDTA reaches its peak concentration in the serum within 1-2 hours after administration⁴⁷, however in constipation-predominant IBS patients who are known to bear with delayed gastric emptying marker absorption may be delayed⁶⁵. In support, there was no significant decrease in the twenty-four-hour ⁵¹Cr-EDTA excretion between IBS-C patients and controls. Dysregulation of epithelial barrier function leads to increased exposure to luminal antigens, bacterial translocation and activation of the mucosal immune system. Of interest, low grade inflammation of the intestinal mucosa, increased number of mast cells, T-cells and

proinflammatory cytokines have lately been verified by several studies on IBS, mostly being present in the ileocecum and in the colon²⁴⁻²⁷. This is in agreement with our results regarding the localization of increased permeability of IBS-D patients. We may also speculate that the release of inflammatory mediators, as a consequence of low-grade mucosal inflammation, is sufficient to sensitise sensory neuron nerve terminals, possibly resulting in a decreased threshold for visceral sensitivity in IBS patients. Until recently, reports are contradictory on correlation between gut permeability and IBS symptoms^{19,20,66}. In our present study, we add new information that among several clinical symptoms evaluated, stool frequency correlates well with colonic permeability in IBS-D patients. Similar correlation between colonic permeability and stool frequency cannot be observed in UC patients with low partial Mayo score, which also supports the theory of different underlying pathophysiology.

Epithelial barrier defect in UC is characterized by three mechanisms: in moderate-to-severe inflammation leaks correlate with epithelial erosions or ulcers and in mild forms leaks are considered to be either foci of epithelial apoptosis or altered epithelial tight junction structure^{67,68}. It is also well-known that neutrophil transmigration across mucosal epithelium is a hallmark of inflammatory conditions, such as IBD⁶⁹. Moreover, neutrophil accumulation within epithelial crypts and in the intestinal lumen directly correlates with clinical disease severity and epithelial injury^{70,71}. Even though cathepsin-G is one of the most abundant proteins found in human and mouse neutrophils⁷², no clinical studies have so far evaluated its involvement in the pathogenesis of IBD. Our present findings show that UC fecal supernatants increased paracellular permeability in murine colon. Pepducin P4pal-10, a lipopeptide that specifically blocks PAR-4 signaling⁷³, abolished the increased paracellular permeability caused by UC fecal supernatants. Moreover, PAR-1 and PAR-2 antagonists had no effect on increased paracellular permeability triggered by UC fecal supernatants. In addition, the synthetic peptide AYPGKF-NH₂, which can activate PAR-4 but no other PARs⁷⁴, was able to reproduce the effect of UC fecal supernatants on CPP in mice. Accordingly, we concluded that fecal supernatants from patients with UC contained an amount of serine-proteases, which is able to activate PAR-4 localized on epithelial cells, to initiate an increase in CPP, a phenomenon that was abolished by a selective cathepsin-G inhibitor. Thus, Cat-G appears to be a major colonic luminal factor present in UC to stimulate PAR-4 to trigger

epithelial barrier alternations in active disease. So far, little information has been available on the mechanism of epithelial barrier defect in UC in remission. Our *in vivo* data have shown that colonic permeability is impaired in inactive UC irrespective of the extension of the disease, when comparing patients with left-sided colitis or pancolitis. This novel finding regarding increased colonic permeability in inactive UC is in agreement with the fact that myosin light chain kinase (MLCK) expression, which is a key enzyme in regulating cytoskeletal contractility and thus tight junction permeability, is also increased in patients with histologically inactive UC⁷⁵. In inflamed mucosa of patients with UC upregulation of pore-forming claudin-2 tight junction protein has been reported, however no such changes were seen in inactive disease^{59,60}. Though little is yet known about structural alterations in the epithelial tight junction in inactive UC and one might speculate that it offers a plausible explanation to the persistent “leaky gut”, it needs further evaluation.

To summarize the new results of the thesis, we have shown for the first time that (i) serine protease activity can be used as a biomarker to distinguish between IBS-D and acute infectious diarrhea, and permits differentiation of IBS-D and UC patients in the absence of increased fecal inflammatory markers; (ii) elevated luminal serine protease activity seen in IBS-D patients is sufficient to trigger an increase in colonic permeability and subsequent visceral hypersensitivity in mice, suggesting similar effects in humans; (iii) this effect of luminal serine proteases on colonic permeability and subsequent visceral hypersensitivity is PAR-2-mediated; (iv) colonic exposure to supernatants of IBS-D patients triggers a rapid phosphorylation of MLC and a subsequent, delayed internalisation of ZO-1 in colonocytes *in vivo*; (v) elevated serine-protease activity seen in UC is able to trigger epithelial barrier disruption; (vi) in active UC, a neutrophil-derived mediator, cathepsin-G is responsible for the barrier disruption able via PAR-4 activation; (vii) impaired epithelial barrier function is localized to the colon and is restricted to the diarrhea-predominant subtype of IBS patients; (viii) increased colonic permeability in IBS-D patients correlates with stool frequency and (ix) that colonic epithelial barrier is also compromised in patients with UC in remission.

In conclusion, these data support an organic background for IBS offering novel therapeutic approaches in the treatment of the disease considering luminal serine protease inhibition or PAR-2 antagonism as valid therapeutic targets. IBS is a highly prevalent functional

gastrointestinal disease whose heterogeneous nature is coupled with a poorly understood pathogenesis, which hinders both patient diagnosis and treatment. Substantial efforts are ongoing in an attempt to bridge the gaps of current knowledge on the pathophysiology of the disease in the hope that the success will ease the diagnosis of IBS and result in more efficient patient management. Considering inflammatory bowel diseases, our data also implicate that there is no complete restoration of epithelial barrier function even in remission of UC. Since cathepsin-G and PAR-4 have been shown to be responsible for epithelial barrier disruption, they may represent a novel, promising therapeutic approach in the treatment of IBD. Even though the question whether altered barrier function makes a primary or secondary contribution to IBS and IBD pathogenesis still persists, restoring barrier function remains a future therapeutic objective.

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ANNEXES I.

ÖSSZEFOGLALÓ

Bevezetés A gyulladásos bélbetegségeket (IBD) és az irritabilis bél szindróma hasmenés-predomináns formáját (IBS-D) magas lumenális szerin-proteáz aktivitás jellemzi. Ismert, hogy a szerin-proteázok proteáz-aktivált receptorokon (PAR) keresztül emelkedett paracelluláris permeabilitás kiváltására képesek. Irodalmi adatok alapján mind az irritabilis bél szindróma (IBS), mind pedig az IBD patogenezisében az epitheliális barrier sérülése szerepet játszhat.

Célkitűzések Vizsgálatainkban célul tűztük ki, hogy (i) meghatározzuk az emelkedett lumenális szerin-proteáz aktivitás eredetét IBS-D-ben, (ii) megvizsgáljuk, hogy az emelkedett szerin-proteáz aktivitás képes-e kiváltani emelkedett vastagbél permeabilitást illetve colorectalis hiperszenzitivitást IBS-D-ben, (iii) tanulmányozzuk a PAR-2 lehetséges szerepét a fenti folyamatokban (iv) illetve, hogy felderítsük az annak háttérében álló molekuláris mechanizmusokat, (v) megvizsgáljuk, hogy a colitis ulcerosa (UC) betegekben tapasztalt emelkedett lumenális szerin-proteáz aktivitás hozzájárulhat-e az emelkedett bélpermeabilitás kiváltásához illetve fenntartásához és (vi) elemezzük egy neutrophil szerin-proteáz, a cathepsin-G illetve a PAR-4 lehetséges szerepét a fenti folyamatban. Továbbá célul tűztük ki, hogy (vii) meghatározzuk IBS-ben és (viii) remiszióban lévő UC-s betegekben a vékony- és vastagbél permeabilitást in vivo, és (ix) megvizsgáljuk, hogy van-e korreláció az emelkedett bélpermeabilitás és a klinikai tünetek között IBS-D-s betegekben.

Betegek és Módszerek Meghatároztuk a széklet szerin-proteáz, a pankreász elasztáz-1, a polimorfonukleáris-elasztáz, a szekretoros leukocita proteáz inhibitor, a calprotectin és a myeloperoxidáz aktivitást egészséges egyénekben, valamint IBS-ben, UC-ben és akut fertőző hasmenésben szenvedő betegekben. A viscerális szenzitivitást kolorectalis ballon dilatációra adott elektromyográfiás válasszal vizsgáltuk kontroll és IBS széklet szupernatánsok intrakolonikus infúzióját követően vad típusú illetve PAR-2-/- egerekben. A vastagbél paracelluláris permeabilitás (CPP) vizsgálata egér vastagbél preparátumokon történt Ussing kamrában. A tight junction fehérje zonula occludens-1 (ZO-1) és a foszforilált myosin könnyű lánc (MLC) detektálása immunhisztokémiával történt. Az in vivo bélpermeabilitás meghatározására per os elfogyasztott ^{51}Cr -EDTA-t használtunk, melynek a vizeletbe történő kiválasztását 24 órán keresztül mértük IBS-ben és UC-ban szenvedő betegekben, valamint

kontroll egyénekben. Az IBS-D betegcsoportban a klinikai tüneteket értékeltük, majd a vastagbél permeabilitáshoz korreláltattuk.

Eredmények IBS-D alcsoportban tapasztalt magas széklet szerin-proteáz aktivitás nem tulajdonítható sem epithelialis, sem pedig gyulladásos eredetűnek, valamint kizárható, hogy csökkent endogén antiprotáz aktivitással társulna. Az IBS-D széklet szupernatáns intrakolonikus alkalmazása allodyniát és emelkedett CPP-t váltott ki egérben, mely jelenségek szerin-proteáz gátlókkal kivédhetők és fennállásuk PAR-2 expresszióhoz kötött. Az IBS-D széklet szupernatáns intracolonikus alkalmazása egyúttal a colonocytákban korai MLC foszforilációhoz vezet, valamint kései ZO-1 internalizációt okoz. UC széklet szupernatáns emelkedett CPP-t vált ki egér vastagbél preparátumon, mely jelenség cathepsin-G inhibitorral illetve PAR-4 antagonistá alkalmazásával kivédhető. In vivo, a proximális vékonybél permeabilitás szignifikánsan csökken az IBS székrekedéssel járó altípusában, ugyanakkor viszont a distalis vékonybél permeabilitás nem mutat szignifikáns különbséget az egyes betegcsoportokban a kontrollhoz képest. A vastagbél permeabilitás IBS-D-ben és remisszióban lévő UC-s betegekben szignifikánsan magasabb a kontroll egyénekéhez képest. Az IBS-D alcsoportban az emelkedett vastagbél permeabilitás a vizsgált klinikai tünetek közül a székletszámmal mutat korrelációt.

Konklúziók Az IBS-D-s betegekben tapasztalt magas lumenális szerin-proteáz aktivitás PAR-2 mediált epithelialis barrier károsodást valamint allodyniát okoz egérben, mely felveti egy új organikus ok lehetőségét az IBS pathogenezisében. Az emelkedett lumenális cathepsin-G aktivitás PAR-4 mediált mechanizmus révén kiválthatja illetve hozzájárulhat az epithelialis barrier sérüléséhez UC-ben. IBS-ben a bélpermeabilitás növekedése az IBS-D betegcsoportra korlátozódik és a vastagbéltre lokalizálható. A barrier diszfunkció remisszióban lévő UC-s betegekben is fennáll, mely szintén a vastagbéltre lokalizálódik. Bár továbbra is kérdés, hogy a megváltozott barrier funkció primer vagy szekunder szerepet tölt be az IBS illetve az IBD pathogenezisében, annak helyreállítása a jövőben is terápiás cél lehet.

ANNEXES II.

ÖSSZEFOGLALÓ

Bevezetés A gyulladásos bélbetegségeket (IBD) és az irritabilis bél szindróma hasmenés-predomináns formáját (IBS-D) magas lumenális szerin-proteáz aktivitás jellemzi. Ismert, hogy a szerin-proteázok proteáz-aktivált receptorokon (PAR) keresztül emelkedett paracelluláris permeabilitás kiváltására képesek. Irodalmi adatok alapján mind az irritabilis bél szindróma (IBS), mind pedig az IBD patogenezisében az epitheliális barrier sérülése szerepet játszhat.

Célkitűzések Vizsgálatainkban célul tűztük ki, hogy (i) meghatározzuk az emelkedett lumenális szerin-proteáz aktivitás eredetét IBS-D-ben, (ii) megvizsgáljuk, hogy az emelkedett szerin-proteáz aktivitás képes-e kiváltani emelkedett vastagbél permeabilitást illetve colorectalis hiperszenzitivitást IBS-D-ben, (iii) tanulmányozzuk a PAR-2 lehetséges szerepét a fenti folyamatokban (iv) illetve, hogy felderítsük az annak háttérében álló molekuláris mechanizmusokat, (v) megvizsgáljuk, hogy a colitis ulcerosa (UC) betegekben tapasztalt emelkedett lumenális szerin-proteáz aktivitás hozzájárulhat-e az emelkedett bélpermeabilitás kiváltásához illetve fenntartásához és (vi) elemezzük egy neutrophil szerin-proteáz, a cathepsin-G illetve a PAR-4 lehetséges szerepét a fenti folyamatban. Továbbá célul tűztük ki, hogy (vii) meghatározzuk IBS-ben és (viii) remiszióban lévő UC-s betegekben a vékony- és vastagbél permeabilitást *in vivo*, és (ix) megvizsgáljuk, hogy van-e korreláció az emelkedett bélpermeabilitás és a klinikai tünetek között IBS-D-s betegekben.

Betegek és Módszerek Meghatároztuk a széklet szerin-proteáz, a pankréasz elasztáz-1, a polimorfonukleáris-elasztáz, a szekretoros leukocita proteáz inhibitor, a calprotectin és a myeloperoxidáz aktivitást egészséges egyéneknél, valamint IBS-ben, UC-ben és akut fertőző hasmenésben szenvedő betegekben. A vizcerális szenzitivitást kolorectalis ballon dilatációra adott elektromyográfiás válasszal vizsgáltuk kontroll és IBS széklet szupernatánsok intrakolonikus infúzióját követően vad típusú illetve PAR-2/- egerekben. A vastagbél paracelluláris permeabilitás (CPP) vizsgálata egér vastagbél preparátumokon történt Ussing kamrában. A tight junction fehérje zonula occludens-1 (ZO-1) és a foszforilált myosin könnyű lánc (MLC) detektálása immunhisztokémiával történt. Az *in vivo* bélpermeabilitás meghatározására *per os* elfogyasztott ^{51}Cr -EDTA-t használtunk, melynek a vizeletbe történő kiválasztását 24 órán keresztül mértük IBS-ben és UC-ban szenvedő betegekben, valamint kontroll egyéneknél. Az IBS-D betegcsoportban a klinikai tüneteket értékeltük, majd a vastagbél permeabilitáshoz korreláltattuk.

Eredmények IBS-D alcsoportban tapasztalt magas széklet szerin-proteáz aktivitás nem tulajdonítható sem epithelialis, sem pedig gyulladásos eredetűnek, valamint kizárható, hogy csökkent endogén antiproteáz aktivitással társulna. Az IBS-D széklet szupernatáns intrakolonikus

alkalmazása allodyníát és emelkedett CPP-t váltott ki egérben, mely jelenségek szerin-proteáz gátlókkal kivédhetők és fennállásuk PAR-2 expresszióhoz kötött. Az IBS-D széklet szupernatáns intracolonus alkalmazása egyúttal a colonocytákban korai MLC foszforilációhoz vezet, valamint kései ZO-1 internalizációt okoz. UC széklet szupernatáns emelkedett CPP-t vált ki egér vastagbél preparátumon, mely jelenség cathepsin-G inhibitorral illetve PAR-4 antagonistá alkalmazásával kivédhető. In vivo, a proximális vékonybél permeabilitás szignifikánsan csökken az IBS székrekedéssel járó altípusában, ugyanakkor viszont a distalis vékonybél permeabilitás nem mutat szignifikáns különbséget az egyes betegcsoportokban a kontrollhoz képest. A vastagbél permeabilitás IBS-D-ben és remisszióban lévő UC-s betegekben szignifikánsan magasabb a kontroll egyénekéhez képest. Az IBS-D alcsoportban az emelkedett vastagbél permeabilitás a vizsgált klinikai tünetek közül a székletszámmal mutat korrelációt.

Konklúziók Az IBS-D-s betegekben tapasztalt magas lumenális szerin-proteáz aktivitás PAR-2 mediált epithelialis barrier károsodást valamint allodyníát okoz egérben, mely felveti egy új organikus ok lehetőségét az IBS pathogenezisében. Az emelkedett lumenális cathepsin-G aktivitás PAR-4 mediált mechanizmus révén kiválthatja illetve hozzájárulhat az epithelialis barrier sérüléséhez UC-ben. IBS-ben a bélpermeabilitás növekedése az IBS-D betegcsoportra korlátozódik és a vastagbéltre lokalizálható. A barrier diszfunkció remisszióban lévő UC-s betegekben is fennáll, mely szintén a vastagbéltre lokalizálódik. Bár továbbra is kérdés, hogy a megváltozott barrier funkció primer vagy szekunder szerepet tölt be az IBS illetve az IBD pathogenezisében, annak helyreállítása a jövőben is terápiás cél lehet.